AD_____

Award Number: DAMD17-99-1-9258

TITLE: Critical Role for Aberrant CpG Island Methylation in the Evolution and Progression of Breast Cancer: Characterization of Known Genes and Identification of Novel Genes

PRINCIPAL INVESTIGATOR: Steven A. Belinsky, Ph.D.

CONTRACTING ORGANIZATION: Lovelace Biomedical and Environmental Research Institute
Albuquerque, New Mexico 87185

REPORT DATE: September 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Annual (1 Sep 99 - 31 Aug 00)

4. TITLE AND SUBTITLE
Critical Role for Aberrant CpG Island Methylation in the Evolution and Progression of Breast Cancer: Characterization of Known Genes and Identification of Novel Genes

5. FUNDING NUMBERS
DAMD17-99-1-9258

6. AUTHOR(S)
Steven A. Belinsky, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lovelace Biomedical and Environmental Research Institute
Albuquerque, New Mexico 87185

E-MAIL:
sbelinsk@LRRI.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

CpG island methylation is an epigenetic modification of DNA associated with the silencing of gene transcription. The hypothesis of this proposal is that breast cancers develop along different pathways, some involving aberrant CpG island methylation for gene inactivation. Some of the genes or CpG islands identified in the methylation-dependent pathways will also be inactivated early in breast cancer evolution and/or will be associated with prognosis. These hypotheses will be tested in innovative studies that will identify novel CpG islands methylated in stage I or III breast cancers using the new technique of methylation specific representation differential analysis. The frequency and timing for methylation of these novel CpG islands will be defined in a case control study of ductal cell carcinoma *in situ*, stage I, and stage III ductal cell carcinomas. This study is collecting data on other risk factors (e.g., hormone profiles, alcohol intake) for breast cancer. In addition, cellular and genetic endpoints that are potentially prognostic for this disease: cell proliferation index (Ki-67), HER2/neu, progesterone receptor and estrogen receptor expression are being determined in this ongoing study. The novel technique of methylation-specific polymersae chain reaction will be used to detect methylated alleles in DNA from fixed tissue. Together, these results will identify novel CpG islands in breast cancer, define their timing for inactivation, identify pathways that may lead to breast cancer through gene inactivation by methylation, and identify markers of prognosis.

14. SUBJECT TERMS
Breast Cancer, CpG island methylation

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

(4) Introduction

The development of breast cancer may involve the inactivation of many different genes potentially resulting in several different "pathways" along which breast cancers evolve. Therapeutic approaches which target these pathways could result in improved survival rates and potentially cures for early stage disease. The goal of this study is to identify novel genes involved in breast cancer and to determine whether pathways exist that target these genes for inactivation. This study will focus on one specific mechanism by which genes are inactivated, the addition of a methyl group to cytosines within the promoter region of the gene, a phenomenon termed CpG island methylation. Aberrant CpG island methylation blocks the expression of the gene, thereby causing it to lose its normal function. Several genes already identified, p16, HIC1, estrogen, and E-cadherin are altered by this mechanism in breast cancer. The studies proposed will be focused on the identification of novel genes which are inactivated in breast cancer through CpG island methylation. The timing for inactivation and whether inactivation is associated with prognosis will also be determined. Novel genes inactivated by CpG island methylation will be identified by a PCR-based technique called methylation specific representational differential analysis (1). The novel PCR fragments will be sequenced and a homology search of GenBank using the BLAST program will be conducted. The methylation state of these novel CpG sequences will then be determined in tumor and normal pairs (n = 15) of both stage I and III ductal carcinoma. If coding sequence can be identified, RNase protection assays will also be conducted on tumor and normal pairs or breast cancer-derived cell lines to confirm that methylation within the CpG island leads to loss of expression. Finally, the frequency and timing for methylation of the novel CpG island containing genes will be defined in a case-control study. A New Mexico Women's Health Study is being conducted within the Epidemiology and Cancer Control Program at the University of New Mexico. This case-control study is enrolling 500 cases (300 non-Hispanic whites; 200 Hispanic whites) throughout New

Mexico and a subset of these samples will be evaluated. Persons eligible for study participation will have a primary diagnosis of carcinoma of the breast (in situ and Stages I, II, and IIIA). This study is investigating potentially modifiable prognostic factors for breast cancer such as hormone status, dietary and alcohol intake, and reproductive history. In addition, cellular and genetic endpoints that are potentially prognostic for this disease: cell proliferation index (Ki-67), HER2/neu, progesterone receptor and estrogen receptor expression are being determined in this ongoing study. Together, these results will identify novel CpG islands in breast cancer, define their timing for inactivation, identify pathways that may lead to breast cancer through gene inactivation by methylation, and identify markers of prognosis.

(5) Body

There have been several changes in the Statement of Work. First, the original scope of work was for 48 months, however; the study section only recommended funding for 36 months since the study was not considered population-based. Therefore, we have decided to focus our efforts on identifying novel CpG islands methylated in breast cancer and to evalute their timing and frequency in samples from the New Mexico Women's Health Study. We have eliminated examining genes known to be methylated in breast cancer (p16, E-cadherin, estrogen receptor α) so that our studies will have the most significant impact with respect to novel and new findings. The second change made was with respect to the technique proposed to identify the novel methylated CpG islands. In the original application, we proposed to use the technique of methylation sensitive restriction fingerprinting developed by Gonzalgo et al. (2) and Huang et al. (3). However, in the interim period between submission of our proposal and funding, our major collaborator's laboratory (Dr. Stephen Baylin, Johns Hopkins) developed another technique (1) that appeared more robust for identifying differentially methylated CpG islands in cancer.

Therefore, we elected to apply this technique to the identification of methylated CpG islands in breast cancer.

The first task proposed (month 1) involved the collection of 15 tumor and normal pairs of stage I and III breast cancers. The collection of these samples through the University of New Mexico hospital is now proceeding efficiently and we have obtained 15 tumor and normal pairs. We expect to obtain another 15 pairs over the next 3 - 6 months.

The second task, to generate the DNA finger-prints, was suppose to occupy the remainder of the first year. Since, we switched strategies to using the methylation sensitive representational differential analysis, significant progress has been made to not only identify candidate CpG islands, but to sequence the DNA (a task for months 13 -15) and to conduct the homology search (task for month 16). Described below is the strategy used in conducting the methylation sensitive representational differential analysis and a description of our progress to date.

The basic concept underlying this technique involves first the amplification by PCR of closely methylated SmaI sites to enrich for methylated CpG islands. Approximately 70 - 80% of CpG islands contain at least two closely spaced (< 1 kb) SmaI sites (CCCGGG). Only those SmaI sites within these short distances can be amplified and this ensures that the most CpG rich sequences will be represented. Briefly DNA is digested with SmaI, which cuts only unmethylated sites, leaving blunt ends between the C and G. DNA is then digested with the SmaI isoschizomer XmaI, which does cut methylated CCCGGG sites, and leaves a 4 base overhang. Adapters are ligated to this overhang, and PCR is performed using primers complementary to these adapters. To identify CpG islands differentially hypermethylated between tumor and normal tissue, these amplification products can be used as templates for subtraction techniques such as representational differential analysis that uses DNA from the tumor as the tester and DNA from the normal tissue as the driver. This is the essence of the procedure that we have elected to use.

In order to avoid any contamination from normal breast tissue, we elected to use the MCF-7 breast cancer cell line as the tester and DNA isolated and pooled from 5 different normal breast tissues as the driver. Both DNA samples were first digested with SmaI and then with XmaI. Two different sets of adapters were ligated onto the DNA fragments. These adapters differ with respect to their GC content and thus, the PCR reactions performed to amplify the DNA fragments are conducted at two different annealing temperatures. This allows one to amplify moderately GC rich sequences (40 -60%) and highly GC rich sequences (60 - 80%). Following the PCR step two different subtractions were done, one with the high GC rich amplicons (tester and driver) and one with the moderately GC rich amplicons. After completion of the first subtraction, the eluted sample was then used as the tester in a second subtraction. Following the second subtraction, the eluted DNA was ligated to pBluescript and transformed into bacteria. After 24 hours, 96 colonies were picked from each transformation (high and moderate GC subtractions).

Plasmid preparations were generated from the 192 colonies and the insert was amplified by PCR using T7 and T3 sequences surrounding the insert. All colonies selected contained inserts that varied in size from approximately 200 - 800 basepairs. Because Alu sequences are often methylated in normal tissue, it is important to first screen the colonies for this repetitive sequence. The PCR products from the 192 colonies were spotted onto nylon membranes and hybridized with a ^{32}P-labeled human Alu repetitive sequence. Sixty of the 192 inserts hybridized to the repetitive Alu sequence and were eliminated from further screening approaches.

Ten inserts from the moderately GC rich substraction library have been further characterized. First, DNA from three breast cancer-derived cell lines, MCF-7 (the tester), MDBA-231, and MDBA-435, and the pooled normal DNA (driver) were spotted onto nylon membranes. All of these DNA samples were first subjected to the digestion protocol, ligation with the two different adaptors, and PCR amplification to generate the putative methylation

specific amplicons. Each of the inserts was labeled with ^{32}P and hybridization to the 3 cell lines and normal tissue was assessed. Five of the inserts hybridized to all breast cancer cell lines and normal tissue indicating a lack of differential methylation. Two of the inserts hybridized selectively only to MCF-7, while three of the inserts hybridized to all 3 of the breast cancer-derived cell lines, but not the normal tissue. These 5 inserts were then subjected to sequencing. The size of these inserts ranged from 370 - 580 base pairs with GC contents of 48 - 60% (indicative of CpG islands).

Database searches were conducted for all 5 of the sequenced inserts and all matched sequence data that had been deposited as part of the human genome project. One of the inserts appears to be in the promoter region of a known gene not previously described in cancer, but proposed to function in the transport and uptake of cholesterol. Using the Gene Scan Analysis Software (MIT, Boston, MA), one of the clones appears to be within a GC region located near a putative promoter that lies upstream of an open reading frame that codes for a predicted protein of approximately 1900 amino acids. Further characterization of the other 3 inserts is ongoing. In addition, studies are in progress to determine the methylation-state of these 5 CpG islands in tumor normal primary breast samples.

(6) Key Research Accomplishments

Collected 15 tumor normal pairs of ductal carcinoma.

Performed methylation sensitive representational differential analysis between MCF-7 and normal breast tissue.

Screened 194 clones from the differential analysis subtraction for Alu repetitive sequence.

Screened 10 clones for their ability to differentially hybridize to DNA from breast cancer-derived cell lines versus normal breast tissue.

Identified 5 clones that appear differentially methylated between cell lines and normal breast tissue.

Sequenced the 5 differentially methylated clones and conducted homology search.

Identified all 5 clones within the database, linked one to a recently characterized gene.

(7) Reportable Outcomes

None to date.

(8) Conclusions

During the first year of this study, we successfully completed the subtraction of two libraries and initated the screening process to identify differentially methylated CpG islands in breast cancer. Five putative islands showing differential methylation between breast tumor-derived cell lines and normal tissue have been identified and are being characterized further. All of these inserts matched sequenced deposited as part of the human genome project, thus greatly facilitating our efforts to evaluate the sequence around our inserts.

(9) References

1. Toyota, M., Ho, C., Ahuja, N., Jair, K-W, Li, Q., Toyota, M., Baylin, S.B., and Issa, J-P J. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res. 59: 2307-2312, 1999.

2. Gonzalgo, M.L., Liang, G., Spruck, C.H., Zingg, J-M, Rideout, W.M., and Jones, P.A. Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. Cancer Res. 57: 594-599, 1997.

3. Huang, T.H., Laux, D.E., Hamlin, B.C., Tran, P. Tran, H., and Lubahn, D.B. Identification of DNA methylation markers for human breast carcinomas using the methylation-sensitive restriction fingerprinting technique. Cancer Res. 57: 1030-1034, 1997.

(10) Appendix

None attached